Genetic introgression and female mate choice within the *Calendulauda albescens/C. barlowi* hybrid zone

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Abstract

The hybrid zone between *Calendulauda albescens* and *C. barlowi*, located near the Orange River, contains a wide range of phenotypically intermediate individuals. This study examines the mitochondrial DNA of some of these individuals to determine if possible introgression is occuring from *C. albescens* to *C. barlowi*. Mitochondrial DNA from 13 lark individuals found in the *C. albescens*/*C. barlowi* hybrid zone was analysed. A 358 base pair cytochrome-b sequence was obtained. The neighbour-joining phylogenetic tree obtained revealed two distinct clades, a *C. albescens* and *C. barlowi* clade. The *C. barlowi* clade was supported with a bootstrap value of 100. The *C. albescens* clade contained individuals of intermediate morphology, as represented by their hybrid index scores, indicating that intermediates between the two species contain the mitochondrial DNA of only one species, *C. albescens*. These results point at introgression from *C. albescens* to *C. barlowi*, with female *C. albescens* choosing to mate with male *C. barlowi*.

Introduction

Hybridization between two taxa may occur in narrow areas, often between the edges of the ranges of the two parental taxa. In many cases, these parental populations are genetically distinct (Barton & Hewitt 1989), but are able to mate successfully to produce offspring that possess a mixture of characteristics of both parents (Barton & Hewitt 1985).

The occurrence of hybrid zones in intermediate areas between the edge of two populations may be due to the fact that hybrids produced through cross species matings are likely to possess characteristics from each parental species that allows them to thrive where the parental populations could not (Burke & Arnold 2001). The fitness of hybrids within these intermediate areas is likely to be higher than that of parental individuals (Lloyd *et al.* 1998), possibly due to advantages of heterozygosity of the genetic stocks from the parental populations (Grant & Grant 1992). However, not all hybrid individuals will experience the same evolutionary fitness as each other, since there is often a large diversity of gene combinations found within hybrid zones (Barton & Hewitt 1989). However, on either side of a hybrid zone, hybrid fitness is expected to be lower as parental populations should be better adapted to the conditions found there (Lloyd *et al.* 1998). Some views however question the viability of hybrid individuals (Burke & Arnold 2001), mainly due to incompatibility between the fusion of two different genetic stocks from contrasting parental individuals (Grant & Grant 1992). Exceptions do however occur, with both hybrid and mixed pairs of two species of *Larus* gulls performing better than pure parental pairs with regard to reproductive success (Good *et al.* 2000).

The occurrence of hybrid zones in animals is generally rare (Grant & Grant 1992), although some studies have focused on grasshoppers, frogs and a few small mammals (see Barton & Hewitt 1985 for references). Birds though are an exception to this, with hybridisation being a relatively common occurrence in birds (Grant & Grant 1992). Birds possess a diverse genetic compatibility that allows morphologically diverse, non-sister taxa to interbreed successfully (Gill 1998). Avian hybridisation studies have amongst others, looked at hybrid zones between the Hermit (*Dendroica townsendi*) and Townsend's Warbler (*D. occidentalis*) (Rohwer & Hewitt 1998),

Larus gull species (Good *et al* 2000) and the Spotted (*Strix occidentalis caurina*) and Barred (*S. varia*) Owls (Kelly & Forman 2004). In this paper, a hybrid zone between *Calendulauda albescens* (Karoo Lark) and *C. barlowi* (Barlow's Lark) is studied.

C. albescens and *C. barlowi* are non-sister taxe found in the Alaudidae family(Ryan *et al.* 1998). The sister taxon of *C. barlowi* has been shown to be the Dune Lark, *C. erythrochlamys* (Ryan *et al* 1998). *C. albescens* and *C. barlowi* differ in their amount of streaking and colour. *C. albescens* is more heavily streaked on its breast and back, and it has streaked flanks (Dean & Ryan 1997, Sinclair & Ryan 2003) whereas those of *C. barlowi* are plain. There are also differences in bill size and plumage colour. The plumage colour of many members of the Alaudidae is similar to that of the sand colour they nest on (Dean *et al.* 1992). Populations of both *C. albescens* and *C. barlowi* occur on a range of sandy habitats, resulting in a range of colour forms (brown to reddish) in both species (Ryan *et al.* 1998). Even with this similarity in colour, the two species are distinctive (C. Cohen, pers. comm.). The habitat types of the two species are similar. *C. albescens* is found in Karoo and coastal scrubland (Ryan *et al.* 1998), as well as perennial grasslands (Dean & Ryan 1997). *C. barlowi* is found between Port Nolloth and Aus in Namibia, occurring in arid scrublands, vegetated dunes (Ryan *et al.* 1998) and in areas of sparse succulent Karoo (Dean & Ryan 1997).

Calendulauda barlowi was first recognised as a species by Ryan *et al.* (1998), based on mitochondrial DNA, plumage, morphology and vocalisations (Ryan *et al.* 1998; Cohen, 1998). Although *C. albescens* and *C. barlowi* are non-sister taxa, intermediate individuals are found in the area between the species' range (Ryan *et al.* 1998). It was predicted that this zone of contact between the two species could in fact be an area of hybridisation between *C. albescens* and *C. barlowi* (Ryan *et al.* 1998). Further investigation using morphological variation suggested that a hybrid zone did in fact exist between *C. albescens* and *C. barlowi* (Cohen, 1998; Cohen & Spottiswoode 2000).

In the past, intermediate morphology was used solely to identify hybrid individuals (Dowling & Secor 1997). With the application of molecular analysis, mtDNA haplotypes of parental populations found in suspected hybrid individuals were able to provide answers as to whether hybridisation was occurring (Rhymer & Simberloff 1996). The development or deeper understanding of potential hybrid zones needs input from other genetic sources. These include allozymes, microsatellite DNA, single-copy nuclear DNA (Rhymer & Simberloff 1996) as well as morphology (Dowling & Secor 1997).

The aim of this study is to analyse the mitochondrial DNA of birds showing intermediate phenotypes found within the apparent hybrid zone of *C. albescens* and *C. barlowi*. Previous work within the hybrid zone noted mixed pairs of female *C. albescens* and male *C. barlowi* (Cohen, 1998). This could result in the introgression of *C. albescens* mitochondrial DNA into *C. barlowi* birds. If this were the case, it is expected that the maternally inherited mitochondrial DNA of hybrid individuals will be the same as *C. albescens* individuals. If mating within the hybrid zone were random, there should be an equal representation of *C. albescens* and *C. barlowi* mitochondrial DNA haplotypes (Dowling *et al.* 1997).

Methods

Sampling

The thirteen birds used in this study were collected from various localities (Fig 1) within the suspected *Calendulauda albescens*/*C. barlowi* hybrid zone, and were chosen to represent as close as possible the greatest variety of phenotypes observed within this area (Cohen, Hons. thesis). Three character states were used to score each of the individuals captured. These characters were back streaking, flank streaking and upperparts colour and were quantified as follows:

1. Back streaking : 0 = none, 0.5 = intermediate, 1 = heavy
2. Flank streaking : 0 = none, 0.5 = intermediate, 1 = heavy
3. Upperparts colour: 0 = sandy, 0.5 = intermediate/brown, 1 = reddish component,

where zero represents a typical *C. barlowi* and one represents a typical *C. albescens* (Cohen, 1998).

Hybrid index

Using the above characters, a hybrid index (HI) was calculated for each bird. This was accomplished using a multivariate principle component (PC1) analysis of the variable characters (Cohen, 1998). The PC1 used by Cohen was calculated by incorporating plumage character scores from 268 larks observed in and around the hybrid zone. This index was scaled from 0, which was a typical *C. barlowi* to 1, a typical *C. albescens* (Cohen, 1998).

DNA extraction and lab procedure

Total genomic DNA was extracted from heart tissue using the DNeasy animal tissue protocol provided with the DNeasy® tissue kit (Qiagen). The mitochondrial cytochrome *b* locus was amplified using primers L14990 (5'-CCATCCAACATCTCAGCATGATGAAA-3') and H15499 (5'-GGTTGTTTGAGCCTGATTC-3'). DNA was amplified using the polymerase chain reaction (PCR). The PCRs contained 3.0µl NH_4 buffer, 3.0µl $MgCl_2$ (25mM), 1.2µl dNTPs (10µM), 1.0µl of each primer, 0.15µl of Super-Therm Taq, 3.0µl DNA template and 17.65µl PCR water to make up 30µl reactions. The thermal profile comprised an initial denaturation step at 94°C for two minutes, followed by 30 cycles of 94°C for one minute, 52°C for one minute and 72°C for two minutes, with a final polymerisation step of 72°C for seven minutes. Amplified products were then cleaned following the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) protocol. In order to sequence the amplified DNA, up to 4µl of the amplified DNA was added to 2µl TRR (Big Dye® Terminator V3.1 cycle sequencing Ready Reaction Kit), 1µl 5x NH_4 buffer, 0.16µl primer and 2.84µl PCR water to create a reaction volume of 10µl. Sequencing products were resolved on an ABI PRISM® 3100 Genetic Analyzer.

Forward and reverse sequences were assembled and checked using SeqManII (LaserGene systems software, DNAstar, inc.). Checked sequences and sequences of pure *C. albescens* and pure *C. barlowi* obtained from GenBank (accession numbers from Ryan & Bloomer 1998) were then manually aligned using MegAlign (LaserGene systems software, DNAstar, inc.).

Phylogenetic analysis

In order to determine the phylogenetic relationship between the 13 sequences used in this study a neighbour joining tree was constructed using PAUP4.0b10 (Swofford 2001) mounted on Apple Macintosh G4. The trees were constructed using Tamura-Nei parameter distances (Cicero 2004). Trees were mid-point rooted. Bootstrap values for the neighbour joining tree were obtained using distance settings using PAUP4.0b10 (Swofford 2001) and 1000 replicates.

Results

Hybrid Index Scores

The hybrid index (HI) calculated for the 13 larks used in this study are shown in Table 1, along with collection sites and character scores. Five of the 13 individuals were of intermediate morphology (HI = 0.31 – 0.70), three are classified as "*C. barlowi*" (HI = 0.00 – 0.30) and five were classified as "*C. albescens*" (HI = 0.71 – 1.00).

Phylogenetic analysis

The length of the cytochrome-b sequence analysed in this study was 358 base pairs. This length was restricted through the incorporation of pure *C. albescens* and *C. barlowi* sequences obtained from GenBank, as sequences generated in this study did not correspond completely with the GenBank sequences. This difference is due to the use of slightly different primers used in the Ryan *et al.* (1998) study that generated the pure *C. albescens* and *C. barlowi* sequences.

The neighbour-joining tree incorporating Tamura-Nei distances (Cicero 2004) and bootstrap and branch length values are shown in Figure 2. Two main mitochondrial DNA haplotypes were found in the specimens sampled, represented by the formation of two distinct clades on the tree (Fig 2). Individuals comprising the *C. barlowi* clade (Larks 4, 12 & 13)(Fig 2; Bootstrap = 100) all possess *C. barlowi* mitochondrial DNA and have very low hybrid index scores (Table 1). Of the 13 intermediate individuals sampled, five fell into the "hybrid" classification (0.31 – 0.70; Table 1) according to the hybrid index (Cohen, 1998). These birds all possessed *C. albescens* mitochondrial DNA, forming a clade along with individuals very similar to true Karoo Larks. It is expected that haplotypes obtained from individuals in this study should be the same as either of the *C. albescens* or *C. barlowi* sequences obtained from GenBank. There are however up to two base pair changes in a few individuals. Lark 13, found within the *C. barlowi* clade (Fig

2), experienced a base pair change at position 53, while lark 9 experienced base pair changes at position 105 and 277. Lark 1 had a single base pair change at position 265.

Discussion

Mixed mating and mate choice

Results from the phylogenetic analysis show the mtDNA make-up of apparent hybrids that are phenotypically intermediate between *C. albescens* and *C. barlowi*. The mitochondrial DNA of these birds is that of *C. albescens* only. Cohen (1998) noted that breeding pairs within the hybrid contact zone consisted mainly of *C. albescens* females and *C. barlowi* males (six out of seven pairs). Although these observations were made from very few mixed pairs, there is an indication of negative assortative mating within the hybrid zone, with female *C. albescens* mating with the slightly larger *C. barlowi* males.

In a review by Randler (2002), it was found that hybridisation mostly occurs through the necessity to mate when conspecifics are rare or through mistaken identity during mate recognition. There are many individuals of *C. albescens* and *C. barlowi* within the Karoo/Barlow's Lark hybrid zone (Cohen, 1998). There is therefore no lack of male *C. albescens* to mate with the female of the species. Even though both *C. albescens* and *C. barlowi* undergo the same colour change as the species move inland (Cohen, 1998), the species are still very distinct.

Hybrid superiority could also be a determining factor of female mate choice within the hybrid zone (Good *et al.* 2000). Female birds have a higher parental investment than male birds and therefore choose to mate with males that will produce offspring with the highest fitness within the hybrid zone (Randler 2002). Avian hybrid zones are located at the edge of species boundaries, an intermediate habitat of the two parental species. Hybrid individuals may therefore be fitter in this intermediate environment as they possess a genome that consists of elements from the genetic stocks of the parental species adapted to the environments on either side of the hybrid zone (Lloyd *et al.* 1998). By choosing to mate with male *C. barlowi* within the hybrid zone, female *C. albescens* birds are able to

ensure that the offspring produced have a higher fitness than any conspecific offspring produced within the hybrid zone.

Hybridisation and introgression

All phenotypically intermediate individuals (HIS = 0.30 – 0.70) possess mitochondrial DNA haplotypes of *C. albescens* (Fig 1; Table 1). If mating between *C. albescens* and *C. barlowi* individuals within the hybrid zone was random, mitochondrial DNA haplotypes of hybrid individuals should be equally represented by *C. albescens* and *C. barlowi* haplotypes (Dowling *et al.* 1997). The lack of equal representation of both parental species' haplotypes indicates that introgression may be occurring between *C. albescens* and *C. barlowi*; with the genes of *C. albescens* moving across into the *C. barlowi* population. Since introgression often only occurs in one direction (Rhymer & Simberloff 1996), it can, in extreme cases, lead to the extinction of the rarer species (Rhymer & Simberloff 1996). Given that all intermediate hybrid individuals sampled in this study, all possessed *C. albescens* mitochondrial DNA, backcrossing could occur into the *C. barlowi* parental population, decreasing the genetic variation found within the species. Although abundant in the area of collection (Cohen, 1998), the distribution range of *C. barlowi* is very small (Ryan *et al.* 1998). The effects of Karoo Lark introgression may therefore move quickly through the Barlow's Lark population.

Since habitat modification is also known to increase the amount of hybridisation between species (Rhymer & Simberloff 1996). Increased changes in the habitat of *C. barlowi's* habitat within its distributional range could explain the occurrence of hybridisation between *C. albescens* and *C. barlowi*. The majority of the range of *C. barlowi's* range falls within mining areas and there are reports of population decline in grazing areas (Dean & Ryan 1998). Since both parental species are found naturally in different habitat types (Dean & Ryan 1998; Sinclair & Ryan 2003), reproduction between the two species may be reduced. Subsequent modification of *C. barlowi* habitat by mining and agriculture may have pushed the species distribution southwards into denser

Karoo vegetation inhabited by *C. albescens*, leading to a breakdown of reproductive isolation (Rhymer & Simberloff 1996) once occurring between *C. albescens* and *C. barlowi*. However, due to the recent recognition of *C. barlowi* as a species (Ryan *et al.* 1998), its historical distribution is not known.

Dowling and Secor (1997) have a more positive view of hybridisation and introgression. With the formation of new genotypes created by hybridisation and introgression, levels of genetic variation increase, providing the possibility for hybrids to respond quickly to environmental changes (Dowling & Secor 1997). Continued hybridisation can lead onto the creation of a new species (Grant & Grant 1997), one that is often unique (Dowling & Secor 1997) and suited to a narrow distribution range that is often the hybrid zone. One of the benefits of observing natural hybrid zones is viewing the evolutionary processes that occur within them (Burke & Arnold 2001). Through observing interactions within the *C. albescens*/*C. barlowi* hybrid zone, mechanisms that cause speciation can be determined.

Whether or not a new species has been formed through hybridisation between *C. albescens* and *C. barlowi*, it is still important to conserve these intermediate individuals. A new genetic stock has been produced, adding genetic variation to the Karoo Lark complex. An increase in genetic variation ensures a higher resistance/tolerance within the species complex. This increased resistance may be important in the near future with climate change models predicting the conversion of the succulent Karoo into desert areas within the next 50 years (Midgley 2001). If species within the Karoo Lark complex are to survive this rapid change of habitat, high amounts of genetic diversity will be needed.

Conclusions

Although the sample size of this study was small (n = 5), there is an indication that female *C. albescens* individuals are choosing to mate with *C. barlowi* males. This may possibly be due to hybrid superiority within the contact zone of these two species. There is also an indication of introgression from *C. albescens* into *C. barlowi*. This may have implications on the genetic variation within the narrowly distributed *C. barlowi* species. MtDNA is able to determine the maternal lineage of hybrid individuals and the direction of introgression, but also needs to be used along side other genetic markers (e.g. nuclear DNA, microsatellite DNA and allozymes) to provide a better idea of the amount of both hybridisation and introgression (Rhymer & Simberloff 1996; Dowling & Secor 1997).

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Table 1: Specimen numbers, collection sites, character scores, hybrid index scores and maternal haplotypes for all lark individuals. See text for details on character score weighting.

Specimen	**Collection site**	**Character scores**			**Hybrid index score**	**Maternal haplotype**
		Back	**Flanks**	**Colour**		
Lark 1	2	2	2	2	0.50	*C.albescens*
Lark 2	4	3	1	3	1.00	*C.albescens*
Lark 3	6	2	3	2	1.00	*C.albescens*
Lark 4	1	1	1	1	0.00	*C.barlowi*
Lark 5	1	3	3	2	0.84	*C.albescens*
Lark 6	2	2	2	2	0.50	*C.albescens*
Lark 7	3	2	2	2	0.50	*C.albescens*
Lark 8	3	3	3	3	1.00	*C.albescens*
Lark 9	4	1	3	2	0.51	*C.albescens*
Lark 10	5	2	2	2	0.50	*C.albescens*
Lark 11	5	3	3	3	1.00	*C.albescens*
Lark 12	8	2	1	1	0.18	*C.barlowi*
Lark 13	8	1	1	1	0.00	*C.barlowi*

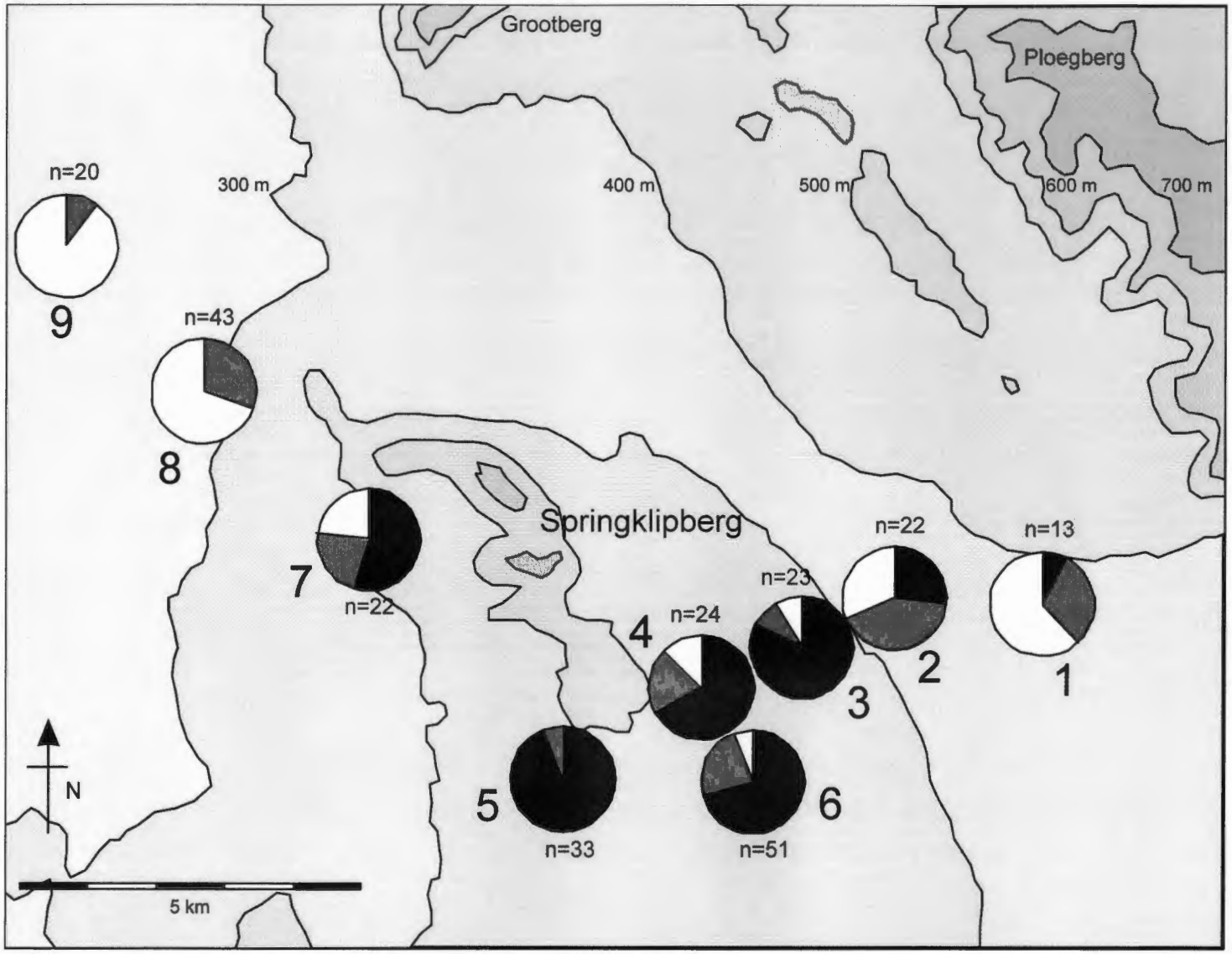

Figure 1: A map showing the sampling localities within the *C. albescens*/*C. barlowi* hybrid zone. Cilcles show the proportion of birds sampled at each location. White = *C. barlowi*, Gray = intermediate and Black = *C. albescens.* (From Cohen, 1998)

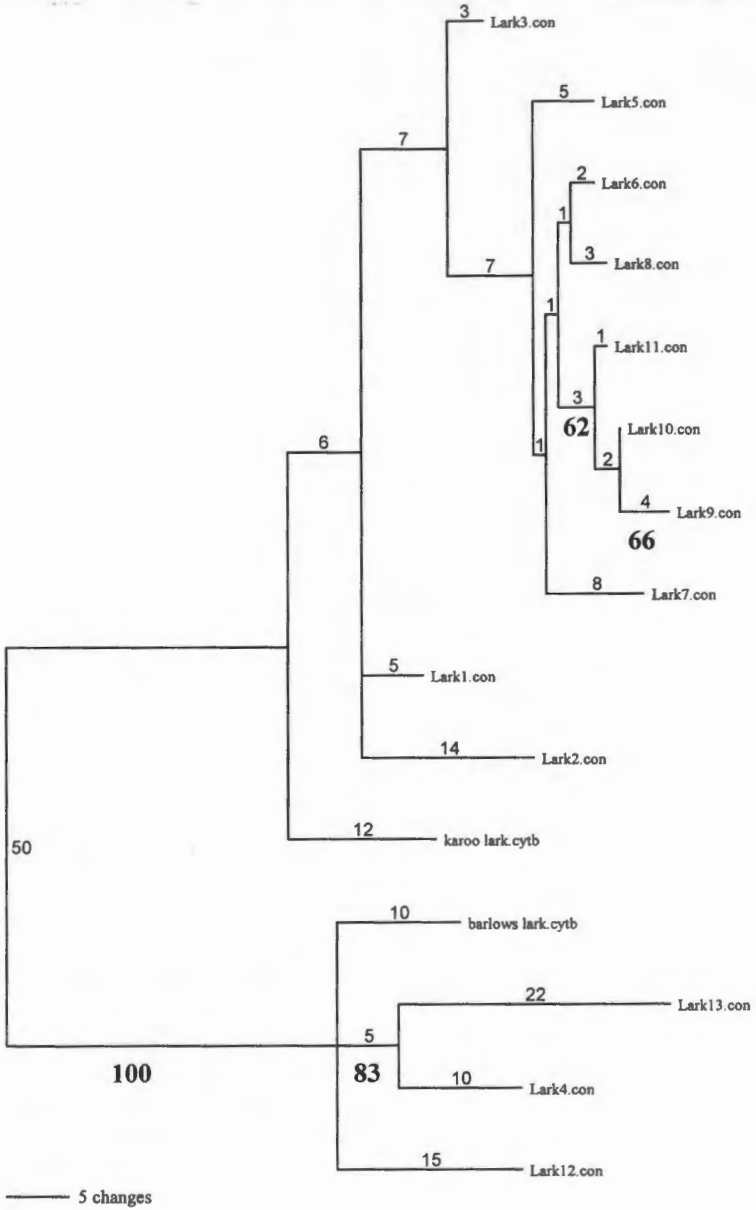

Figure 2: Neighbour joining tree of cytochrome b sequences from 13 lark individuals found within the *C. albescens*/*C. barlowi* hybrid zone along with 'pure' Barlow's and Karoo lark sequences from GenBank. Numbers above branches are branch lengths & those below are bootstrap values.